UNIVERSIDADE DE LISBOA

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DEPARTAMENTO DE BIOLOGIA ANIMAL

Indirect Genetic Effects of Oxytocin in the Development of Social Behaviour in Zebrafish

Diogo Miguel Gonçalves Ribeiro

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Dissertação orientada por:

Prof. Doutor Rui F. Oliveira e Prof. Doutora Susana Varela

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RESUMO

Perceber de que forma os conspecíficos influenciam um único indivíduo e como é que as interações que são estabelecidas entre indivíduos ocorrem em resposta a alterações do ambiente social é ainda um desafio no campo das neurociências e do comportamento. As interações entre indivíduos, ou seja, comportamento social, encontram-se presentes e estendem-se a um vasto número de espécies, desde as mais simples às mais complexas. São estas interações que permitem aos animais percecionarem informação do ambiente social, influenciando assim o *fitness* do próprio e dos indivíduos com que estabelecem ligações produzindo efeitos significativos no decorrer do desenvolvimento e no processo evolutivo e filogenético.

Da forma em que o ambiente social influencia o *fitness* dos indivíduos, o comportamento social pode evoluir por diversas razões: (1) pode promover vantagem na taxa de sobrevivência; (2) reduz a probabilidade de transmissão de doenças e parasitas; (3) evoluir devido a efeitos de competição reprodutiva entre indivíduos do grupo. As interações estabelecidas entre organismos sociais e a sua adaptação conferem-lhes efeitos significativos como proteção contra predação, procura de recursos ou parceiros, além de permitir uma redução dos custos de energia. Esta adaptação dos indivíduos ao ambiente social, nomeadamente plasticidade fenotípica permite que os animais enfrentem os diversos obstáculos adaptando-se às condições pré-existentes. A plasticidade fenotípica é um mecanismo importante na evolução das espécies que permite o aumento das interações sociais e com isto aumentar o *fitness* do indivíduo e da própria espécie.

A ponte principal de comunicação entre animal e ambiente é o comportamento e é com esta função inapta que os animais respondem a novas situações modificando o seu comportamento levando a adaptações morfológicas, psicológicas e que se traduzem numa linha cronológica de eventos importante da vida e desenvolvimento dos indivíduos. A recolha de informação do ambiente é uma ação comum a todos animais desde os mais simples aos mais complexos e que se expressa desde cedo no desenvolvimento podendo ter ações importante para a sua vida. O ambiente além de modificar o comportamento, modifica também um conjunto de mecanismos como por exemplo o cérebro que adapta o comportamento ao ambiente social.

Como mencionado anteriormente, o comportamento dependente essencialmente das interações que são estabelecidas entre outros e estas interações levam a que os animais se adaptem ao ambiente. Resumidamente a variação fenotípica exercida nos animais deve-se a duas componentes, ambiente social e efeitos genéticos (i.e., efeitos genéticos indiretos e diretos), que promovem uma larga variabilidade hereditária. Focando nos efeitos genéticos indiretos, estes descrevem a influência dos genes expressos por conspecíficos no fenótipo do indivíduo focal e deste modo o ambiente cria uma rede genética que poderá levar a alterações genótipo-fenótipo alterando o processo evolutivo.

Sabe-se que são diversas as moléculas envolvidas na regulação do comportamento social e que modulam a sua resposta. Nomeadamente, a oxitocina, um nonapéptido envolvido na criação de ligações entre macho e fêmea (*pair bonding*) e cuidado parental em humanos estende-se a outros vertebrados como peixes e aves. A isotocina, homóloga à oxitocina em peixes encontra-se envolvida na modulação de comportamentos sociais complexos como comportamento de cardume e preferência social. A oxitocina e os seus homólogos são secretados no cérebro e entram na corrente sanguínea através do sistema hipotálamo-neuro-hipofisário, um sistema conservado em todos os vertebrados em que alterações nos seus componentes encontram-se ligados a diversas desordens neurológicas. A produção de oxitocina ocorre nos neurónios parvocelulares e magnocelulares na área pré-ótica.

Diversos estudos demonstraram a importância e função da oxitocina e dos seus recetores na regulação da cognição e comportamento social. Em ratinho, é sabido que mutações na oxitocina promovem deficits sociais como reconhecimento social e memória.

Tendo por base o grande fator que o ambiente social acarreta no desenvolvimento do comportamento nos animais, o presente trabalho centra-se nos efeitos do ambiente social, nomeadamente, o efeito da oxitocina no desenvolvimento das capacidades cognitivas do peixe-zebra. Como tal foi usada uma linha mutada para o recetor da oxitocina que por eliminação de um aminoácido impede a ligação da oxitocina ao recetor, impedindo deste modo a sua libertação.

De modo a avaliar a performance cognitiva, foram realizados três testes comportamentais: reconhecimento social, preferência social e aprendizagem social. Uma vez que este estudo se centra no ambiente social, foram formados quatro grupos (1) 1 wild-type + 5 wild-type; (2) 1 knockout + 5 knockout; (3) 1 wild-type + 5 knockout; (4) 1 knockout + 5 wild-type. Cada grupo com seis larvas foi formado com apenas quatro dias, uma vez que, entre o quinto e o sexto dia os indivíduos começam a adquirir capacidades olfativas e visuais. Desta forma, o ambiente é específico do grupo em que o indivíduo se encontra inserido e não por outros fatores externos.

Entre os três e os quatro meses, os indivíduos realizam os testes comportamentais sendo necessário a realização de genotipagem à priori de modo a distinguir nos grupos de tratamento o indivíduo focal dos parceiros sociais. Relativamente ao reconhecimento social, este permite analisar a capacidade de os indivíduos conseguirem discriminar um conspecífico estranho de um conspecífico familiar recorrendo à memória de curto prazo, capacidade esta adquirida desde o desenvolvimento inicial. A preferência social é o segundo teste realizado e visa testar componentes de aproximação e afastamento social, e por último a aprendizagem social testa a capacidade de os indivíduos adquirirem informação do ambiente e usarem-na de modo a otimizarem o seu *fitness*.

Os testes foram gravados com auxílio a câmaras e os vídeos analisados com um programa de vídeo-tracking (Ethovision®, Noldus). Dos resultados obtidos, diferentes medidas foram analisadas. Para o reconhecimento social foram calculados índices de preferência e de tempo de exploração; na preferência social foi determinado o tempo passado junto aos parceiros sociais; por último na aprendizagem social foi medido o número de escolhas corretas bem como o tempo de latência.

Na preferência social, os dados revelaram a primeira evidência dos efeitos genéticos indiretos nos mutantes de oxitocina. Estes, quando num ambiente social de wild-type apresentam um comportamento semelhante ao do grupo controlo sugerindo uma reversão do fenótipo. Quanto ao wild-type no ambiente knockout o mesmo não se observa sugerindo que diferentes ambientes apresentam diferentes forças seletivas nos indivíduos.

Os resultados revelaram que apenas os animais wild-type, independentemente do ambiente, conseguiram discriminar um conspecífico estranho de outro familiar passando mais tempo junto ao indivíduo estranho mostrando assim evidência de memória a curto prazo. Uma vez que o protocolo foi realizado três vezes, os resultados sugerem também a existência de memória a longo prazo com um espaçamento temporal de 48 horas.

Por último a aprendizagem social não mostrou nenhuma evidência uma vez que a taxa de escolhas corretas se encontrou abaixo dos 50% indicando uma não aprendizagem. Tal resultado pode ter-se devido ao facto de os demonstradores encontrarem-se no mesmo lado a uma distância relativamente curta, não acarretando nenhum custo-benefício para o indivíduo focal a investigação de um ou de outro.

Por último através de câmaras com vista de cima e de lado foi testada a dinâmica de grupo, uma vez que diferentes ambientes promovem diferentes interações entre os indivíduos. Gravações de 10 minutos permitiram analisar a coesão do grupo revelando que os mutantes da oxitocina apresentam uma maior coesão de grupo enquanto o ambiente wild-type torna o grupo mais disperso. Através da análise da coesão do grupo do indivíduo wild-type no ambiente knockout, os dados sugerem que basta um único indivíduo para modificar a dinâmica interna do grupo.

Palavras-chave: Comportamento social, efeitos genéticos indiretos, oxitocina, peixe-zebra

ABSTRACT

Understanding how conspecifics influence individual behaviour and how these interactions occur in response to changes in the social environment is a major challenge in social neuroscience. Social behaviour is dependent on the interactions that animals establish between them. These interactions may influence the fitness of other individuals, and have profound effects in their life history and on the evolutionary process. In fish species, behaviours such as antipredator response, enhancement of foraging activity, mating opportunity and also the presence of dominance within the groups are influenced by the social context. Indirect Genetic Effects (IGE) describe the influence of the social partners´ genes on the phenotype of a focal individual, providing a tool to describe interactions in the social environment. Oxytocin-like peptides have been implicated in the regulation of social behavior across taxa, affecting a diversity of behaviours across functional contexts. It is well known that oxytocin and its homologue isotocin affect pro-social behaviour and influence the modulation of complex behaviour. Using the Zebrafish, *Danio rerio* as a model organism, this study focused on the indirect genetic effects induced by oxytocin-like peptides, mainly by assessing the performance of zebrafish of different genotypes (WT vs. $OXTR^{-/-}$) raised in different social environments (WT groups vs. $OXTR^{-/-}$ groups) in different social behaviour paradigms, such as shoal preference, social recognition and social learning, and also by measuring group cohesion; relevant variables that may influence the Darwinian *fitness*. The results suggest an effect of the social environment in the focal individual's behaviour (IGE). When analyzing group cohesion, different dispersion index revealed different social values in the environment suggesting that some environments provide more benefits than others. Thus, we have shown that social environment is a major factor in the development of social behavior, and that social features can revert phenotypes induced by specific genes ($OXTR^{-/-}$).

Keywords: Social behaviour, indirect genetic effects, oxytocin, zebrafish

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ABBREVIATION LIST

Dfp	Days post-fertilization
IR	Infra-red
KO	Knockout
KO C	Knockout control group
KO T	Knockout treatment group
NaOH	Sodium hydroxide
OT	Oxytocin
OXT	Oxytocin-like peptides
OxtR	Oxytocin receptor
ROI	Region of interest
SL	Social learning
SR	Social recognition
SP	Shoal Preference
TL	Tupfel long fin
Tris-HCl	Tris hydrochloride
WT	Wild-type
WT C	Wild-type control group
WT T	Wild-type treatment group

CHAPTER 1 INTRODUCTION

1. Social Environment

In all social systems, animals need to interact to survive in a manner to thrive in their social and physical environments. Social behaviour, which refers to interactions among individuals of the same species, has been documented in a large number of species, from the simplest to the most complex animal taxa (Chapman and Krause 2008; Kalueff et al. 2013; McGlynn 2010).

Animals tend to aggregate and any interaction between individuals that may influence the fitness of other individuals can be regarded as social and such social interactions have profound effects in the life history and on evolutionary process (Trubenová and Hager 2014). When individuals aggregate, they interact whit other group members and different social compositions may improve individual fitness under specific environmental conditions (Philippe et al. 2016). Studies in fish have demonstrated that the density of the social environment, and different social compositions, influence shoaling behaviour and learning with conspecifics (Chapman and Krause 2008).

Diverse social systems have evolved repeatedly across phylogeny during the course of evolution, reflecting adaptations to the environment. Social behaviour may have evolved due to three reasons: (1) it may enhance the original advantage of group living; (2) it reduces the likelihood of disease and parasite transmission; (3) it acts upon the reproductive competition of group members, in relation to other group members, and to the relevant portions of the population (Hamilton 1964).

Behaviour in social species is fundamentally dependent upon interactions with others, frequently with their conspecifics. Living in social groups confers benefits to group members, such as protection against predation, locating of food or mates and also allows a reduction of energetic costs of movement (Chapman and Krause 2008; Oliveira 2013). These advantages rely on behavioural flexibility, an adaptation that allows the animal to face daily changes in social environment (Oliveira 2013; Taborsky and Oliveira 2012). The ability to adapt to changes in the social environment is an important driving force of evolution and such ability will enhance social interactions and thus raise Darwinian fitness (Kotrschal and Taborsky 2010; Oliveira 2013; Spence et al. 2008).

Since behaviour is the key interface between an animal and the environment, when environmental changes occur, animals will respond first through behavioural changes, whereas adaptations in morphology, physiology and life history will arrive subsequently. Animals observe social interactions carefully to gather information that then guides their future behaviour (Fernald 2015). The social environment is a crucial factor on the development of animal social behaviours and can have a crucial influence during early development and throughout their lifes (Arnold and Taborsky 2010). For example, it is well known that in fish, early social environment can be important for social choices (i.e., antipredator behaviour, migration, foraging) and for finding appropriate mating partners during adulthood (Moretz, Martins, and Robison 2007).

Social behaviour not only influences all the aspects that occur during the development of an individual, but also influences the brain to shape the cognitive skills that ultimately modulate the behaviour (Fernald 2015). This modulation relies in two main systems, mesolimbic reward system and social brain network, that together form the social decision-making network. According to this network, the mesolimbic reward system is responsible for the assessment of the relative value of the social stimuli and the consequences of behaving in unrelated forms (O'Connell and Hofmann 2011). The social brain behaviour network is involved in the regulation of multiple forms of behaviour, especially aggression, sexual behaviour, and parental care, which are fundamental and evolutionarily ancient properties of most animals, a major determinant of an individual's fitness.

2. Indirect Genetic Effects on Behaviour

The social environment along with the genetic component leads to phenotypic variation and the IGEs arising from different social environments will provide a large heritable variation. The inheritance and evolution of social behaviour remains a major paradox for evolutionary biologists and behavioural ecologists.

Although the behaviour is expressed by single individuals, their interactions with social partners is very important since early stages of development, and social partners can guide individuals towards important stimuli. It is through these interactions with social partners that social behaviour expressed by a focal individual may change in magnitude and form (interacting phenotypes) and the phenotype of one individual act as an environment for another individual (Bleakley and III 2009; Moore, Brodie, and Wolf 1997; Moretz et al. 2007).

Indirect genetic effects describe the influence of genes expressed by conspecifics on a focal individual's phenotype and thereby provide a framework for understanding the inheritance of traits expressed in social contexts (Moore et al. 1997; Trubenová and Hager 2014).

The influence of indirect genetic effects can be observed when the behaviour of a focal individual responds to changes in the genetic component of the social environment (Bijma 2014; Bleakley and III 2009). Indirect genetic effects can have profound effects on both the magnitude and the direction of response to selection (Bijma 2014). Changing the strain with which focal individuals interacted, it is to directly manipulate the genetic component of the social environment.

In the literature, it has been shown the effect of social environment (IGEs) in behaviours like antipredator and aggression. Regarding the antipredator behaviour, it has been demonstrated that environment can influence group cohesion depending on different predation regimes, the presence of indirect genetic effects influenced focal individual behaviour (Bleakley and III 2009), and on aggression, a study performed in mice shown the role of genes in the environment, and how the genetic component plays an important role in setting the evolutionary potential for aggression (Wilson et al. 2009). The presence of indirect genetic effects in both studies demonstrated that individual's fitness could be shifted in populations with different social environments.

The interactions among social partners, creates a genetic network where the traits expressed by an individual are influenced, and may alter the genotype-phenotype relationship, changing the evolutionary process (Wolf et al. 1998). Therefore, environment computes several mechanisms which control the organism interactions with other individuals, which are extremely dependent on the cognitive abilities of each animal (Zuberbuhler and Byrne 2006).

3. Cognitive Abilities in Group Living Animals

Animals, through the entire taxa, present different cognitive skills that can help them to respond quickly and effectively to any challenge in order to present the adequate behavioural output response. It is through the environment, and the interactions that the animals establish between them that individuals gather social information and identify the cognitive abilities underlying social skills (Kotrschal and Taborsky 2010; Oliveira 2013). Social cognition includes among other skills: recognition of individuals, social partners' preferences, and learning from others conspecifics.

Approach and avoidance is a basic behaviour among animals and is critical for an individual to interact with conspecifics; these interactions between individuals from the same group may raise different difficulties/opportunities leading to competition inside the group, mating or even anti-predator response (Oliveira 2013). The ability of shoaling in fish is acquired early in the development (larval stage) but shoal preference is only exhibited in the juvenile stage (Engeszer et al. 2007). These

preference acquired in early developmental stages is strong and stable, and it is maintain even if social environment changes (Engeszer et al. 2007; Moretz et al. 2007).

The shoal preference which is maintained during the life history of individuals even when the environmental conditions change is due to the capacity that individuals have to discriminate between conspecifics and heterospecifics, and also between conspecifics with different social ranks (Oliveira 2013). This recognition in zebrafish is mediated by a process of phenotype matching against a template based on early experience. The template is acquired through visual and olfactory cues during the larval stage between day 5 and 6 dpf with a significant kin preference (Gerlach et al. 2008; Hinz et al. 2013; Spence et al. 2008). One specific study has demonstrated the importance of early exposure to social environment to the innate capacity of fish to be with conspecifics (shoal preference) and their learned ability to distinguish shoals with different pigment pattern (social recognition). They reported that zebrafish can recognition conspecifics from heterospecifics (Engeszer, Ryan, and Parichy 2004). This recognition has been demonstrated in other studies with different animal models, such as mice and voles where they can discriminate between novel and familiar conspecifics.

Collecting information from the social environment is common to most social animals, and helps them to learn about the environment without paying the costs associated with learning by trial and error. Social learning depends on the social dynamics of the group and occurs in any situation in which the individual can learn by observing the behaviour of others (Coussi-Korbel and Fragaszy 1995; Gale 1996; Galef and Laland 2005). However, social learning is not only doable by social observation, other animals also use different strategies to collect information such as imitation, observational learning of novel foraging techniques, peer or parental influences on individual preferences, as well as outright teaching. It has been demonstrated in adult fruit fly that learning is also important in reproduction where females strongly prefer to lay eggs on food substrate already occupied by larvae (Durisko, Anderson, and Dukas 2014). In lizards the learning ability is age-dependent and a study shown that in early stages, juvenile lizards may be more likely to benefit from social information (Noble, Byrne, and Whiting 2014). Regarding fish, several studies demonstrated learning capacities: (1) social foraging learned from demonstrators where individuals associate a colored chamber with food (Chapman and Krause 2008); (2) shoaling has been described also as a learned social behaviour by Gerlai and colleagues where fish sees a shoal as a reward (Al-Imari and Gerlai 2008).

Shoal preference, social recognition and social learning are complex social skills that are influenced by the environment, and triggers the modulation of the brain and gene expression in order to adapt to new conditions (Al-Imari and Gerlai 2008; Braida et al. 2012).

4. The Relevance of Oxytocin-like modulation in Social Behaviour

Why do some species show complex social behaviours, while others species living in similar ecologies spend the majority of their lives in solitude? This remains a main question that still has not been cleared. It is known that social opportunities produce rapid changes in gene expression in the brain and these genomic responses may prepare the individual to modify their behaviour and adapt to the new social condition (Fernald 2015; Toth and Robinson 2010). Furthermore, different studies have shown that the neuropeptides oxytocin (OXT) and vasopressin (AVP) in mammals, and their homologues in other non-mammalian vertebrates (isotocin in fish, mesotocin in birds, reptiles and amphibian, and arginine vasotocin), are involved in the regulation of social behavior across vertebrates. Several studies have demonstrated that administration of OXT or OXT-like peptides stimulates pair bonding and parental care (Reddon et al. 2012) in humans, and pro-social behaviours not only in humans but also in other vertebrates, such as fish and birds (Eaton and Glasgow 2007). In fish, isotocin is involved in the modulation of complex social behaviours such as, social approach

response, shoaling behaviour, social preference and fear response to predator (Braida et al. 2012; Langen et al. 2015). The oxytocin system is highly pleiotropic, affecting a diversity of behaviours across functional contexts. One possible explanation for this functional diversity is that oxytocin may be involved in a higher-order regulatory system with downstream effects. More recently, it has been proposed that OXT has a role as a central modulator of attention to social stimuli (Reddon et al. 2012).

It is though the hypothalamo-neurohypophyseal system (HNS), a conserved system in all vertebrates, that the OXT-like neuropeptides are released into the bloodstream, but most of the behavioural effects produced by these neuropeptides are due to their central action in the brain. This interface between HNS and the neurovascular system is regulated early in the development by local release of OXT and changes in this system have additionally been linked to several neuropsychiatric disorders (Eaton and Glasgow 2007; Gutnick et al. 2011). OXT production occurs in magnocellular and parvocellular neurons in the preoptic area and these neurons primarily project to the posterior pituitary where OXT is released into the systemic circulation (Eaton and Glasgow 2007).

Several studies have demonstrated the implication of oxytocin and oxytocin receptors in the regulation of social cognition and social behavior. In mice OT null mutants presented social deficits like social recognition and memory (Ferguson et al. 2000).

5. The Zebrafish (*Danio rerio)*

Zebrafish (*Danio rerio*) is a small freshwater teleost that rapidly emerged as an important model organism for neuroscience and behavioural biology to study complex social phenotypes. The main reason for this is that zebrafish are highly social animals, forming multimember groups with structured social relationships (shoals, dominance, hierarchies, exploratory behaviour, social preference) and these behaviors can be easily quantified (Oliveira 2013; Pham, Raymond, and Hester 2012; Spence et al. 2008). In addition, Zebrafish shows a very flexible (behavioural flexibility) leading to changes in connectivity in key nuclei (Kotrschal and Taborsky 2010). This flexibility of social behaviour has been shown in behaviours such shoaling which appear early in their development (Engeszer et al. 2007).

It is known that OXT-like neuropeptides influence the social behaviour in many species and the interactions between individuals (Reddon et al. 2012). Particularly, isotocin, expressed early in the developing brain of Zebrafish leads to an increase in social preference and reduction of fear to predator (Braida et al. 2012; Eaton and Glasgow 2007). By changing the relationship between genotype and phenotype within a population, the existence of IGEs may alter the evolutionary responses, and rates and trajectories of traits expressed in social interaction (Bleakley and III 2009).

6. Aims of the Thesis

Social behavior is a dynamic trait since its expression depends not only on the individual but also on the responses of conspecifics. Therefore, when looking into the mechanisms underlying its expression it is important to focus on both factors. However, most studies addressing the neural and genetic mechanisms of social behavior so far have mainly focused on the individual (e.g. direct genetic effects of candidate genes) and ignored to a large extent variation originating from the social environment. Since social environments are composed by conspecifics their genotypes may also have an effect on the expression of social behavior in a focal individual (i.e. indirect genetic effects, IGE).

Despite the rich literature on the role of OXT on the regulation of social behavior across vertebrates, so far only direct genetic effects have been documented, and the potential role of indirect genetic effects have not been studied. In this thesis, I will investigate both direct and indirect genetic effects of the OXT receptor, using zebrafish (Danio rerio). Zebrafish is a highly social model organism

in which social behaviours are easily phenotyped in laboratory, and mutant and transgenic lines are already available to study the loss of function of OXT-like neuropeptides and their receptors. This strategy will allow to assess the effect of social partners' genes on focal individual social behaviour. It is predicted that if IEGs occur, focal individuals of one genotype raised in social groups of the other genotype should express differences in social behavior from those raised with in social groups of their own genotype. Thus, I aim to document how the social environment (genotype) shapes the brain and alters the behaviour of animals as they interact.

CHAPTER 2 MATERIALS AND METHODS

1. Animal Housing

In this study, a total of 48 zebrafish, *Danio rerio* of both sex, were used in different social environmental contexts. All fish were kept in life support systems at a group size of 6 fish per 3.5 L tank under a 14h light/10h dark cycle, and temperature maintained at 28ºC, pH 7.0 and conductivity at 750 μS/cm.

Once the groups were composed with different social environments at a group size of 6 fish, and to restrict the recognition only to the group, all the experimental groups were formed at 4 days post-fertilization since it has been described that after this age they are capable to imprint for olfactory kin and visual recognition (Gerlach et al. 2008; Hinz et al. 2013).

2. Rearing Conditions

The target gene studied here is the OXT receptor. A single nucleotide deletion leading to a truncated receptor line has been generated by collaborators (Weizmann Institute) using a TALEN-Based Genome Editing system (**Figure 2.1**).

To study both the effect of the genotype, and the effect of the social environment (here the genotype) on the development of social behavior four experimental treatments were used (**Table 2.1**): (1) wild type individuals raised in wild type groups (i.e. group of 6 $OxtR^{+/+}$); OXTR mutants raised in OXTR mutant groups (i.e. group of 6 $OxtR^{-/-}$); wild type individuals raised in OXTR mutant groups (i.e. group of 1 $OxtR^{+/+}$ and 5 $OxtR^{-/-}$); and OXTR mutants raised in wild type groups (i.e. group of 1 $OxtR^{-/-}$ and 5 WT $OxtR^{+/+}$).

Table 2.1. Sample size and abbreviation for each experimental treatment.

Experimental Treatments	*Sample Size*
WT C (6 $OxtR^{+/+}$)	15
KO C (6 $OxtR^{-/-}$)	15
WT T (1 $OxtR^{+/+}$ and 5 $OxtR^{-/-}$)	9
KO T (1 $OxtR^{-/-}$ and 5 WT $OxtR^{+/+}$)	9

3. Genotyping

The social behaviour of the focal fish was assessed at the age of 3–4 months. Before behavioral experiments, fish were genotyped in order to distinguish the focal fish from their social partners on the treatment groups. For the genotyping protocol, the individuals were anesthetized with tricaine (MS-222, 1x) and a small portion of the fin was clipped in order to extract genomic DNA. For individuals within the same group, fin-clipping was performed in different fins (caudal, dorsal, anal) and with different combinations (caudal and dorsal, caudal superior and anal, etc…). Fin clips were collected in a microcentrifuge tube containing 50 µL of NaOH 50 mM (Meeker et al. 2007). The sample was incubated at 95ºC for 20 minutes, placed on ice and the pH adjusted with 1/10th volume of Tris-HCl (1 M, pH 8.0). The genomic DNA was then added to a PCR mix composed of specific primers designed around the oxt-like receptor deletion site (forward 5'-TGCGCGAGGAAAACTAGTT-3' and reverse 5'-AGCAGACACTCAGAATGGTCA-3'). Then, the PCR product was loaded in a 1% agarose gel where one band corresponding to a 700bp product was

expected. The agarose bands were cut from the gel, the DNA was purified/cleaned using a commercial kit (NucleoSpinGel® and PCR Clean-Up (Macherey-Nagel) and sent for sequencing (**Figure 2.1**).

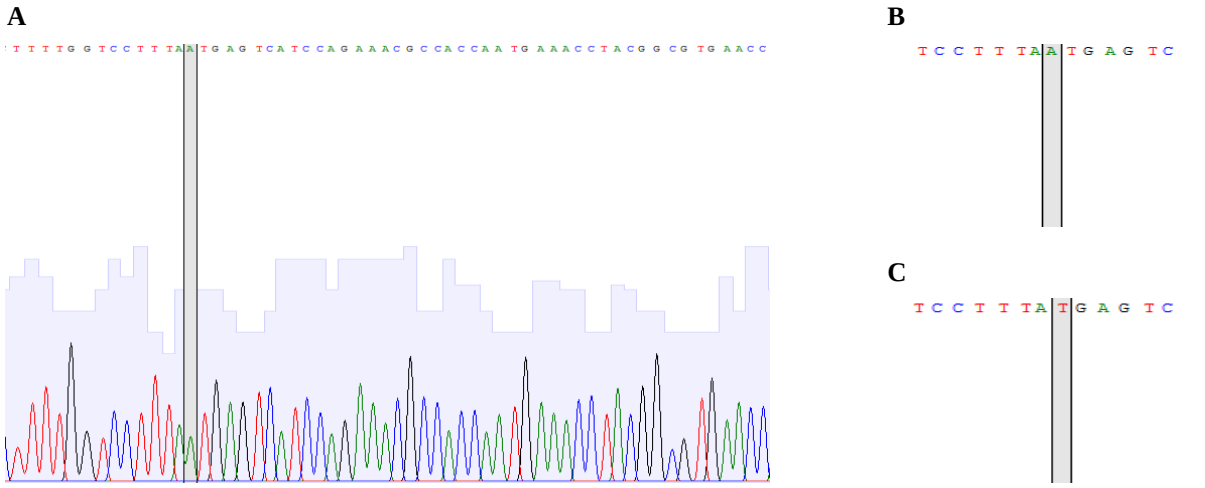

Figure 2.1. Chromatograms of OXT receptor genomic DNA extracted from fish fin. (**A**) Chromatogram of a WT fish, each peak represents a nucleotide which is evenly-spaced without noise. (**B**) Sequence of a WT fish with no deletion of adenine nucleotide. (**C**) Sequence of a OXT receptor mutant with a single nucleotide deletion.

4. Behaviour Tests

Although the life support system allows a high percentage of fish survival some mortality was observed and only focal fish in groups with three or more fish were tested. These tests assessed the three aspects of social behaviour: social preference, social recognition, and social learning. Tests were performed consecutively (morning, afternoon, next morning) and repeated three times (i.e. 3 replicates for each test) to test for the consistency of the results. In each trial, to eliminate the hypothesis of size bias the position of conspecifics was changed. The tanks allocated in the fish facility were transferred to behaviour room one week before the beginning of the behavioural tests to habituate fish to the new conditions. Within one week all fish were habituated to bloodworm and food device used in the tests. During the test days fish were only allowed to eat during the social learning task.

The behaviour tests were recorded through fixed IRs cameras placed above the experimental tanks, which were placed over an infrared lightbox, to increase the contrast between the background and the focal fish. All social recognition and social learning tests were performed between 9:00 and 14:00 and social preference tests were performed between 14:00 and 18:00. The experimental tank consisted in a glass aquarium (30x15x15 cm) and one (in the social learning test) or two (in the social recognition and in the social preference tests) adjacent stimulus tanks (12x12x15 cm), divided into two compartments; **Figure 2.2**). The water depth was kept constant at 9 cm.

The water in the experimental tanks had the same temperature and conductivity as the life support systems and was replaced after each animal had been tested to discard any olfactory cues.

4.1. Social Recognition

The social recognition test is a binary test that evaluates the ability of the focal fish to discriminate between two conspecifics (novel and familiar). By definition, this test also evaluates the short-term memory given the time interval between the tests. It was divided in two steps, in the first step two stranger fish were placed in the stimuli tanks (one on each tank) at each end of the experimental tank where the focal fish was placed. After 10 min of habituation the opaque partitions

that visually separated the experimental tanks from the two top end tanks were removed and the focal fish was able to observe and approach both stimuli fish. After 10 min, the opaque partitions were placed back, and the two strangers removed. In the second step, one of the strangers from the first step of the experiment (now familiar) was placed back in one of the stimulus tank, whereas a third stranger fish was placed in the other stimulus site. After 5 min for habituation the opaque partitions were removed again and the test took 10 min. Social recognition was operationalized as the proportion of time near the target ROI (preference score; **Equation 2.1**) and social exploration was assessed by the time the focal fish spend in both ROIs (exploration times; **Equation 2.2**).

Equation 2.1

$$Preference\ score = \frac{T_{Target\ ROI}}{T_{ROI1} + T_{ROI2}}$$

Equation 2.2

$$Exploration\ Time = \frac{T_{ROI1} + T_{ROI2}}{Total\ Time}$$

4.2. Shoal Preference

This test was performed after the social recognition, in the same day. This task allows to measure approach and avoidance to the shoal, a measurement of sociality. The setup consisted of three tanks (**Figure 2.2**A): a central arena and two stimulus tanks (one in each opposite side). One of the stimulus tank contained a shoal (the same 5 fish that the focal fish was raised with) while the other was empty. After 10 min in a starting box for habituation, fish were allowed to explore the stimuli while video-recorded for 10 min. Shoal preference was measured by the time that the focal fish spent in the ROI near the shoal (**Equation 2.3**).

Equation 2.3

$$Shoal\ Preference = \frac{T_{Shoal}}{T_{Shoal} + T_{No\ Shoal}}$$

4.3. Social Learning

The social learning was tested the day after the other two behavioural tests and aims to test the ability of learning from conspecifics. The set-up consisted in two tanks (**Figure 2.2**B). In the stimulus tank, divided by an opaque partition there were two stranger conspecifics (demonstrators): in one side in the presence of food, and in the other side without food (but in the presence of an empty food container). The test tank, where the focal fish was placed, was divided by a removable opaque, followed by a transparent partition.

During the test, between stimuli and test tanks there was a one-way mirror that allowed the focal fish to observe the other two conspecifics without being seen by them; during the habituation phase, there was also an opaque partition between the stimuli and test tank. The demonstrators and the focal fish were placed in the respective tanks for 10 min to explore the apparatus. After this time of habituation, the focal fish was in zone A and the opaque partition of the test tank was placed back (**Figure 2.2**B). The food containers in the stimuli tanks (one with bloodworm and the other empty) were inserted just before the opaque partition between the stimuli and focal tank was removed, such that when it was removed the focal fish saw one demonstrator eating and the other not. After 5 minutes the focal was in zone B and the opaque and transparent partitions were placed back. The demonstrators were removed and the food containers inserted in the test tank. The opaque partition

was lifted and after another 15 seconds the transparent partition was also removed and the focal fish allowed to choose the location of the food. The test lasted 10 minutes.

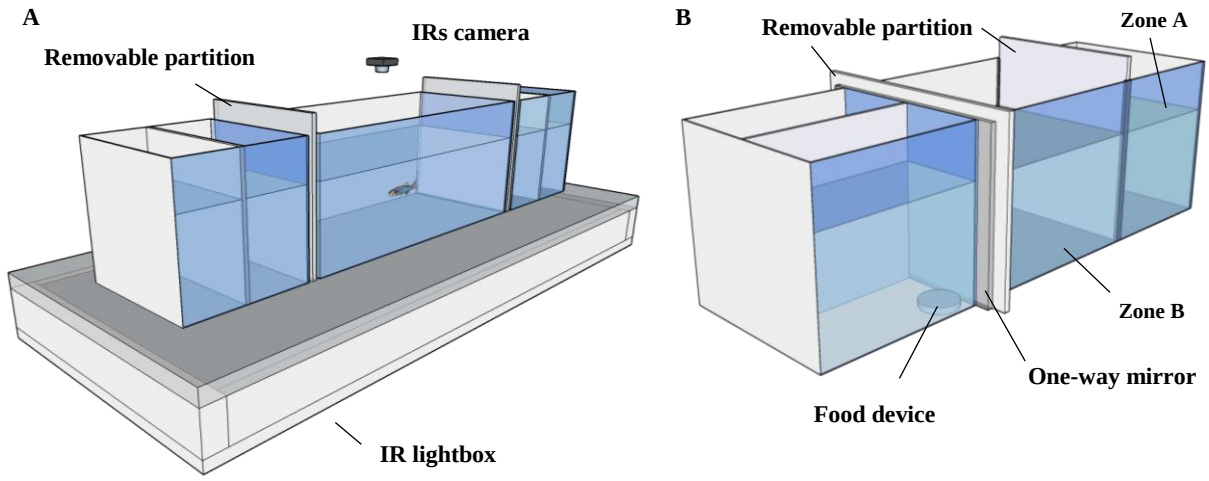

Figure 2.2. 3D diagram of the experimental setup. In both setups fixed IRs cameras recorded all behavioural tests from above. The tanks were placed over an infrared LED custom built lightbox, to increase contrast between the background of the tank and the focal fish. (**A**) 3D diagram of the experimental setup for Social Recognition and Shoal Preference. (**B**) 3D diagram of the experimental setup for Social Learning. One-way mirror allows only focal fish to see demonstrator.

5. Fitness Measures and Group Dynamics

Different social environments may produce different selective forces in the focal individual in large part due to the interactions he experiences with its social partners. To study how the genotype and the social environment influenced the fitness of the individuals three measures were taken two weeks after the behaviour tests: 1) Group cohesion, 2) Reproductive capacity, 3) Body and relative gonadal weight (gonadosomatic index = weight of gonads over body weight).

To study the strength and how the IGE influence individual fitness, group dynamics were recorded in the home tanks by side view with Logitech HD webcam C255, for 10 minutes with eyeline surveillance software (www.nchsoftware.com/surveillance/) to synchronize the cameras. Automated macros developed for the free software ImageJ 1.51d (https://fiji.sc/) allowed us to quantify the shoaling behaviour and analyze the internal dynamic of association among fish. From the group dynamics, we can extrapolate the spread of the group by indexes of fish group dispersion. Group cohesion was analyzed as the sum of the perimeters of each group. A group with higher dispersion index was more spread when compare with a group with lower index that was more aggregated (**Equation 2.4**) (Israeli and Kimmel 1996; Sadoul et al. 2014).

Equation 2.4

$$P_{tot} = \sum_{i=1}^{n} P_i$$

The reproductive capacity was assessed by the mating success, fecundity success, and viability of the embryos. After recording the group cohesion, the focal fish was placed in a breeding box with an individual from the opposite sex with a transparent partition between them. The day after, when the lights turn on the partition was removed and mating allowed for 4 hours. After this period fish returned to their home tanks. If eggs were laid, they were collected and placed in a petri dish with E3 embryo medium and incubated at 28ºC. The next day the viability of the embryos was measured.

The crosses were done outside the life support system at room temperature, however the temperature presented oscillations during the crosses, which may have influenced the results.

Finally, the focal fish was euthanize by incubation in ice water, and dissected (Gupta and Mullins 2010), the gonads were removed and the weighted.

6. Video Tracking

All behavioural tests were recorded through fixed IRs camera at 30 fps rate connected to a laptop using the video recording software, Pinnacle Studio 14 (http://www.pinnaclesys.com/). The group dynamic was recorded using an additional webcam with same frame rate, for an easily fish identification and with the software describe above. The analysis of the behaviour videos was done with the video-tracking software, Ethovision XT11 by Noldus (http://www.noldus.com/animal-behavior-research/products/ethovision-xt), and group dynamic with ImageJ 1.51d ((https://fiji.sc/).

7. Analysis

Regarding the behaviour tests, normality was performed with Shapiro Wilk's. Comparisons of social recognition and shoal preference were assessed with repeated measures ANOVA, followed by Unequal N HSD post-hoc test. Planned comparisons of LS means were performed.

For group cohesion, the effects of genotype and social environment were assessed with an ANCOVA, comparing the slopes and intersections of the linear regressions from control and treatment groups.

All descriptive statistics was reported as mean ± SEM. Statistical significance was set at $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***) and $P < 0.0001$ (****). All statistical analysis was performed with STATISTICA 13 (https://software.dell.com/products/statistica) and graphs built in GraphPad Prism 6 (http://www.graphpad.com/scientific-software/prism/).

CHAPTER 3 RESULTS

1. Social Recognition

In order to test for social recognition, it was assessed if zebrafish could discriminate between novel and familiar conspecifics, social recognition was measured by comparing preference and exploration scores for control and treatment fish (see Methods and **Figure 2.2**A)

1.1. Preference Scores

As described in the literature for other model species, like rats (Ferguson, Young, and Insel 2002), adult zebrafish can discriminate between novel and familiar conspecifics using only visual cues (**Figure 3.1**A). In the first part of the recognition test, the focal WT control fish does not have a preference when faced with two stranger conspecifics. This holds true whether the focal fish is a WT, OXT receptor mutant or has a different genotype from the rest of his group. However, in the second part of the test, when the fish is allowed to discriminate between a familiar and a novel conspecific, WT control fish shows a significant higher preference towards novel fish (**Figure 3.1**A; $F_{(1, 88)} = 2.338$, $P < 0.01$), as well as the wt fish raised among oxt receptor mutant fish, while the Oxt receptor mutant control fish and the oxt receptor mutant raised among wt fish does not have a preference for familiar versus novel fish. Furthermore, in the second part of the test, when the focal fish was exposed to a novel and a familiar conspecific, there was a main effect of Test 2 (**Table 3.1**; Novel > Familiar, Unequal N HSD post-hoc test, $P < 0.01$) where only OxtR WT, independently of the social environment, was able to discriminate between novel and familiar conspecifics (**Figure 3.1**; (**A**): $F_{(1, 88)} = 10.97$, $P < 0.001$; (**C**): $F_{(1, 88)} = 6.947$, $P < 0.05$).

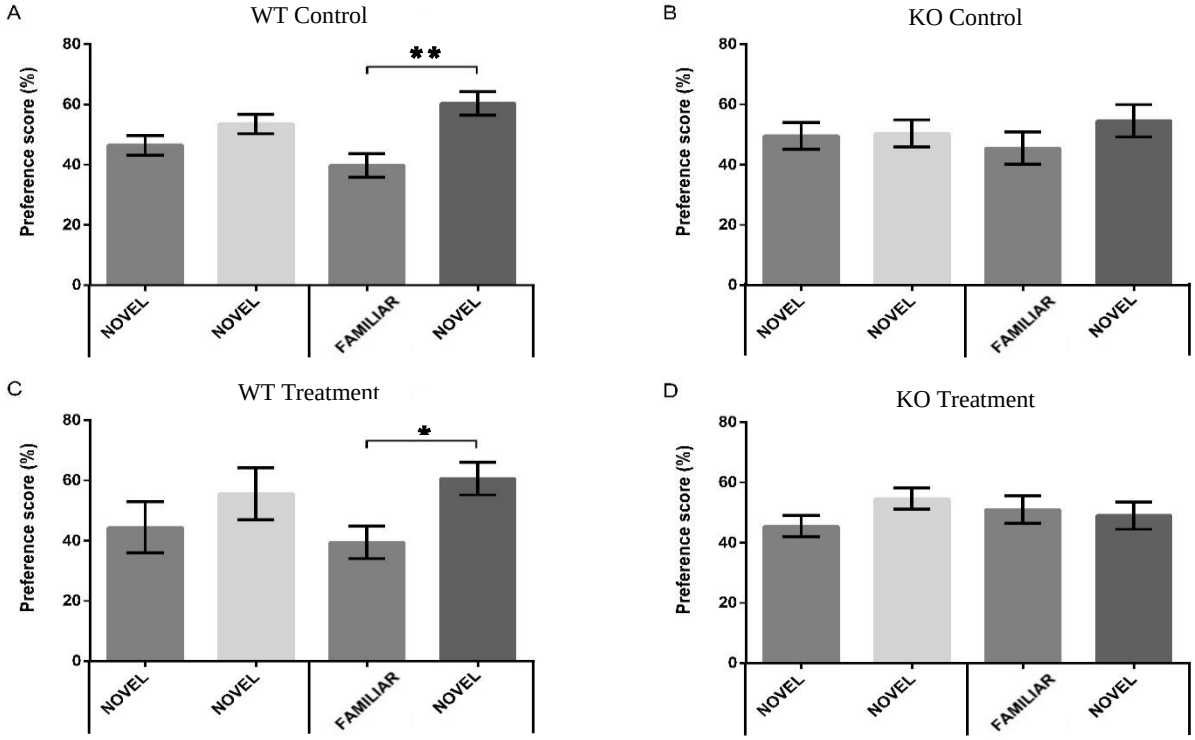

Figure 3.1. Graphical representation of social recognition behavioural test. Preference scores of the time spent near novel-novel (Test 1) or novel-familiar (Test 2) conspecifics. Error bars represent as mean ± SEM. (**A**) OxtR WT control group discriminate between novel and familiar conspecific spending signicantly more time near novel (**B**) OxtR KO control group do not discriminate between novel and familiar. (**C**) OxtR WT treatment group can discriminate between novel and familiar and as the control group spend more time near the novel. (**D**) OxtR KO treatment group do not discriminate between novel and familiar conspecifics. * P < 0.05, ** P < 0.01, *** P < 0.001.

Table 3.1. Effect of social environment on social recognition task. Main effects, interactions and planned comparisons of LS means for Test 1 and Test 2 were calculated using Repeated measures ANOVA. WT C, WT control; KO C, KO control; WT T, WT treatment; KO T, KO treatment.

Effect (Novel – Novel)	*F*	P-*value*
Environment	0.167	0.685
Test 1	0.045	0.833
Environment * Test 1	0.377	0.542

Planned Comparisons (Novel – Novel)

WT C	1.316	0.255
KO C	0.018	0.895
WT T	1.949	0.166
KO T	1.319	0.254

Effect (Novel – Familiar)

Environment	0	1
Test 2	8.002	< **0.01**
Environment * Test 2	2.572	0.059

Planned Comparisons (Novel – Familiar)

WT C	10.97	< **0.001**
KO C	2.134	0.148
WT T	6.947	< **0.05**
KO T	0.063	0.803

In social recognition paradigms, usually only one trial is performed to avoid confounding effects of habituation. In this experimental procedure two additional trials were done and in these following trials the data suggest a spatial long-term memory in OxtR WT individuals in control and treatment groups what was not observed in OxtR KO fish (see **Figure S1**; **Table S1**). In trial 2 the individual spent more time near the tank where in the previous trial the novel conspecific was (**Figure S1**; (**A**): $F_{(1, 88)} = 22.223$, $P < 0.001$; (**E**): $F_{(1, 88)} = 4.587$, $P < 0.05$). In trial 3, in test 1, the focal fish spent more time near the stimulus where in the previous trial it presented the higher preference score (**Figure S1**; (**A**): $F_{(1, 88)} = 7.207$, $P < 0.01$; (**E**): $F_{(1, 88)} = 13.839$, $P < 0.001$).

1.2. Exploration Time

Regarding the exploration times, no differences between the first (Test 1) and the second (Test 2) part of the test was observed in control and treatment groups, which exhibited similar exploratory behaviour (**Table 3.2**; $F_{(1,88)} = 0.257$, $P > 0.05$).

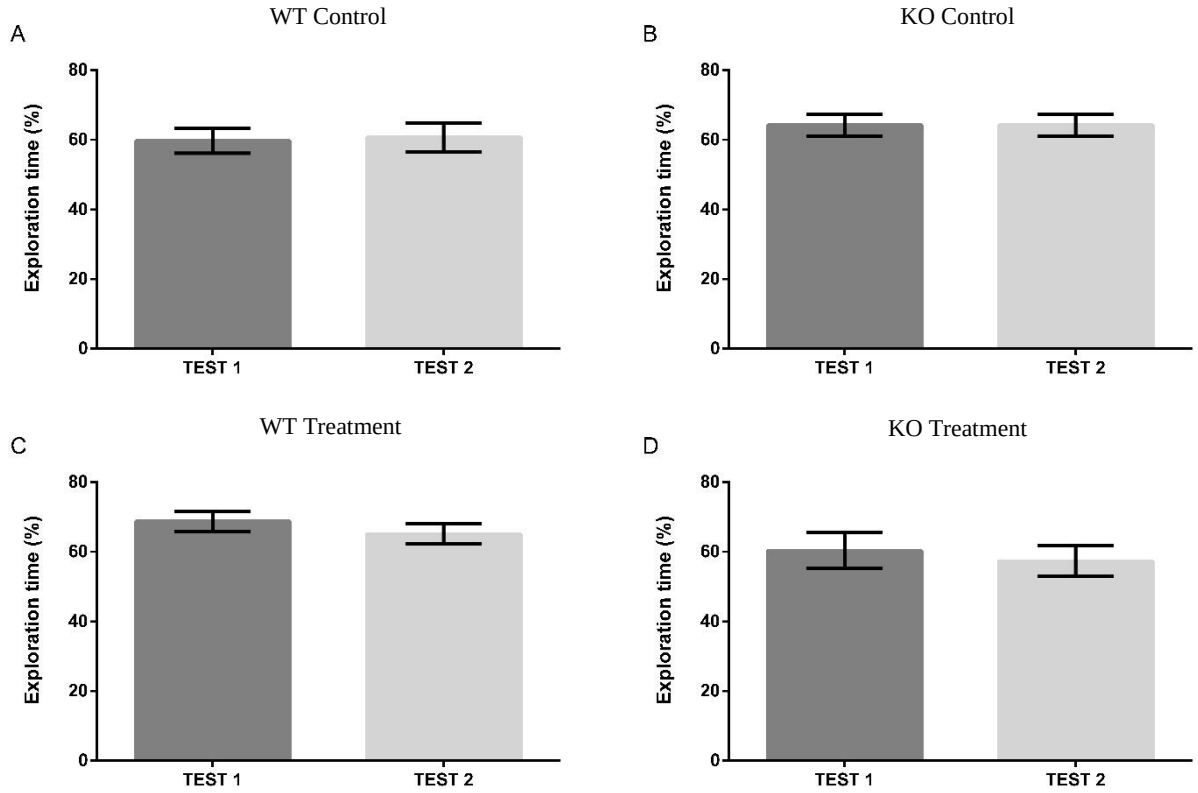

Figure 3.2. Graphical representation of exploration time. Exploration time near each ROI during Test 1 (novel-novel) and Test 2 (novel-familiar). Error bars represent as mean ± SEM. In Test 1 and 2 no differences between exploration time was observed in (**A**) OxtR WT control, (**B**) OxtR KO control group, (**C**) OxtR WT treatment group and (**D**) OxtR KO treatment group. * P < 0.05, ** P < 0.01, *** P < 0.001.

Table 3.2. Effect of social environment on exploration task. Main effects and interactions of Environment and Test were calculated using Repeated measures ANOVA.

Effect	*F*	P-*value*
Environment	1.037	0.380
Test	0.501	0.418
Environment * Test	0.206	0.892

Exploration time in social recognition paradigms, similar to preference scores discussed above, is usually measured only in one trial. With three trials, there was a main effect in Trials with a decrease of exploration time from the first to the last trial (**Figure S2**; Trial 1 > Trial 2 > Trial 3, $F_{(2,176)} = 4.315$, $P < 0.05$) that can be explained by habituation to the experimental setup. There was also a main effect of Trials * Environment (**Table S3**; $F_{(6,176)} = 3.11$, $P < 0.01$) where planned comparisons revealed significant difference between Trial 1 and Trial 3 in OxtR WT treatment group ($F_{(1,88)} = 16.572$, $P < 0.001$).

2. Shoal Preference

In order to assess the cognitive ability of fish to approach or avoid a group of conspecifics, shoal preference was analyzed for 10 minutes, in the experimental setup (see Methods and **Figure 2.2**A). Social scores for both groups, control and treatment were calculated.

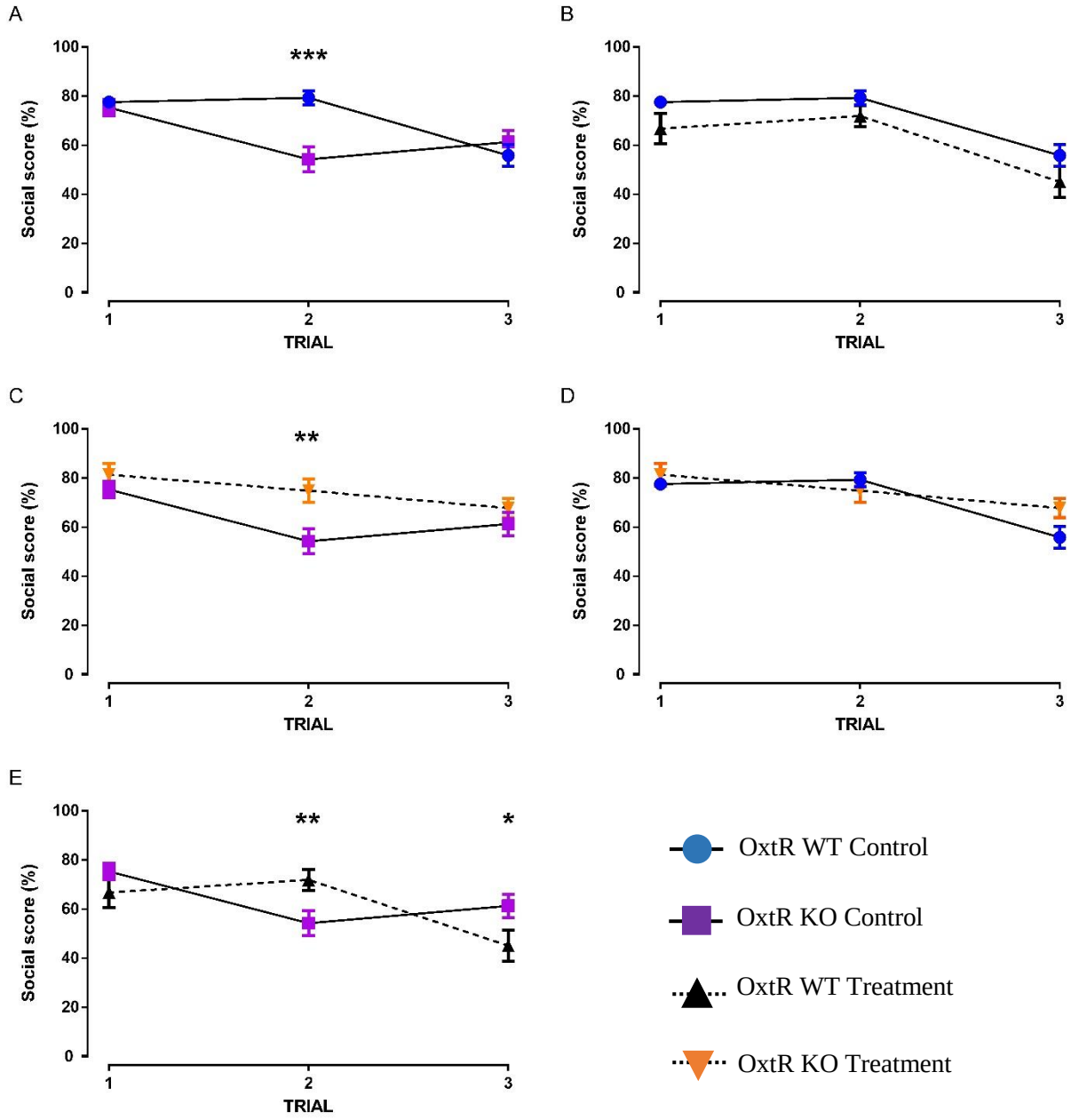

Figure 3.3. Graphical representation of shoal preference behavioural test. Social scores of the time spent in the ROI per trial. Error bars represent as mean ± SEM. (**A**) Genotype presents an effect in shoal preference, OxtR WT control group (n = 15 per treatment) and OxtR KO control group (n = 15 per treatment) presents a significant difference of time spent near the shoal. (**B**) OxtR WT control group and OxtR WT treatment group (n = 9 per treatment) presents a high social score which is maintained until trial 2. (**C**) OxtR KO control group and OxtR KO treatment group (n = 9 per treatment) presents a significant difference of time spent near the shoal in trial 2. (**D**, **E**) The WT environment promotes a higher social score. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Table 3.3. Shoal preference. Main effects, interactions and planned comparisons of LS means were calculated using Repeated measures ANOVA. WT C, WT control; KO C, KO control; WT T, WT treatment; KO T, KO treatment.

Effect	*F*	P-*value*
Genotype	1.36	0.249
Environment	15.39	< **0.001**
Genotype * Environment	0.07	0.795
Trials	16.37	< **0.001**
Trials * Genotype	8.13	< **0.01**
Trials * Environment	0.49	0.614
Trials * Genotype * Environment	1.30	0.277

Planned Comparisons

WT C – KO C	Trial 1	0.225	0.638
	Trial 2	20.802	< **0.001**
	Trial 3	0.772	0.384
WT C – WT T	Trial 1	3.871	0.056
	Trial 2	1.351	0.252
	Trial 3	2.199	0.145
KO C – KO T	Trial 1	1.18	0.283
	Trial 2	10.586	< **0.01**
	Trial 3	0.801	0.376
WT C – KO T	Trial 1	0.456	0.503
	Trial 2	0.485	0.49
	Trial 3	2.742	0.105
KO C – WT T	Trial 1	2.423	0.127
	Trial 2	7.772	< **0.01**
	Trial 3	5.036	< **0.05**

Evidences in literature suggest that oxytocin is an important modulator of pro-social behaviours, such as social approach (Braida et al. 2012; Reddon et al. 2012). As observed in **Table 3.3**, the analysis revealed a main effect of the environment (**Table 3.3**; WT > KO, $F_{(1,44)} = 15.387$, $P < 0.001$) and a main effect of Trials (**Table 3.3**; Trial 3 > Trial 1 = Trial 2, Unequal N HSD post-hoc test, $P < 0.05$). There was also an interaction between two main effects of Trials * Genotype (**Table 3.3**; Trial 1 = Trial 3 (KO > WT), Unequal N HSD post-hoc test, $P < 0.001$). Planned comparison analysis revealed a significant decrease for social preference of OxtR KO control group in trial 2 when compared with OxtR WT control group (**Figure 3.3**; (**A**): $F_{(1, 44)} = 20.802$, $P < 0.001$). When focal fish was raised in a different social environment (i.e. treatment groups, either a WT focal fish raised among mutants or a mutant raised among WTs), in both cases, the social preference score for the focal in the first two trials was higher than the controls. OxtR KO treatment group when compared with the control group presented higher social score (**Figure 3.3**; (**C**): $F_{(1, 44)} = 10.586$, $P < 0.01$).

3. Social Learning

In social learning paradigms using a binary choice, as the one tested here, successful learning is indicated by a percentage of correct choices (above 50%). Zebrafish from both treatments did not learn from conspecifics, since the percentage of correct choices was always below 50%.

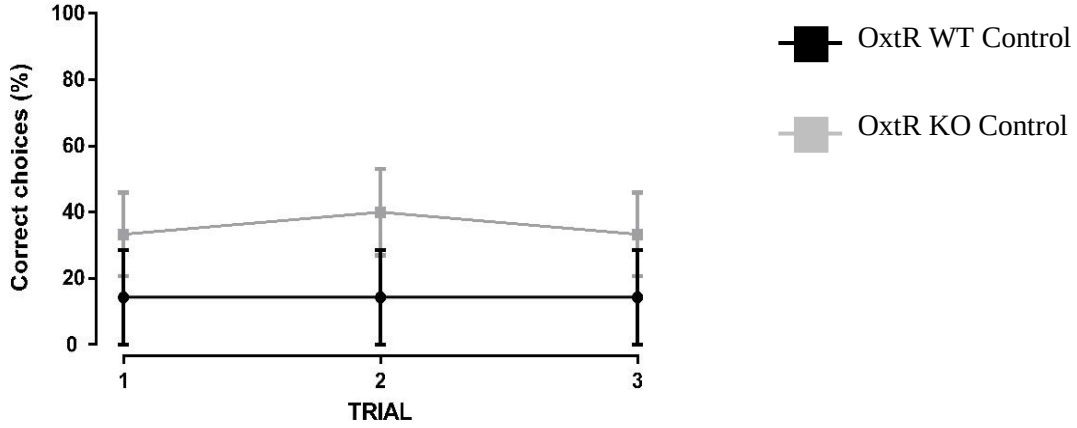

Figure 3.4. Graphical representation of social learning behavioural test. Both WT and KO control groups (n = 15 per treatment did not learn with the conspecifics and no significant difference was observed between them.

4. Group Cohesion

The shoaling behaviour present in many fish species plays a key role in foraging, predator avoidance and mating. To study the temporal dynamics of shoal organization in zebrafish, and how the environment can influence this behaviour, side and top view of the home tank was recorded and analyzed for 10 minutes, in time bins of 6 seconds, for group cohesion.

Regarding the group cohesion of both control groups, the analysis revealed a non-significant difference in the trend of group dispersion along time ($F_{(1,1912)} = 0.038$, P = 0.845), however, the WT control group was significantly more spread then the KO control group ($F_{(1,\ 1913)} = 1923.66$, $P < 0.0001$, **Figure 3.5**A).

For the effect of environment in group cohesion, the analysis revealed a non-significant different in the trend of group dispersion along time in the WT ($F_{(1,1698)} = 0.009$, $P = 0.926$) and KO ($F_{(1,1669)} = 0.894$, $P = 0.345$) environment. In WT environment (**Figure 3.5**B), WT control group had a significantly higher group dispersion when compare with KO treatment group ($F_{(1,1699)} = 305.3$, $P < 0.0001$). For KO environment (**Figure 3.5**C), there was a significant difference between control and treatment group, where the aggregation was higher in KO control group ($F_{(1,1670)} = 814.8$, $P < 0.0001$).

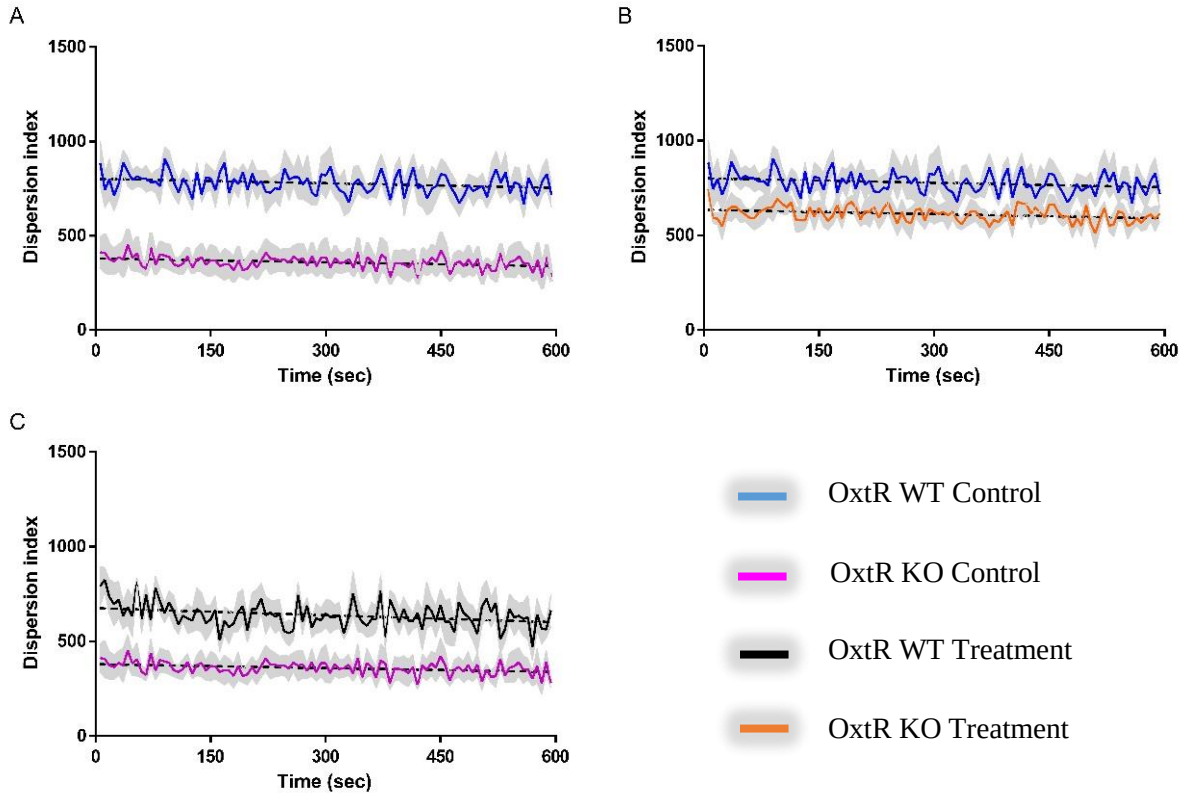

Figure 3.5. Graphical representation of group cohesion. (**A**) Effect of environment in control groups. (**B**) Effect of WT environment in WT control group and KO treatment group. (**C**) Effect of KO environment in KO control group and WT treatment group.

Table 3.4. Multiple linear regression comparisons. Slope gives the temporal variation in group cohesion in the 10 minutes' analysis, and intersect the average group cohesion. Comparisons were calculated with ANCOVA. WT C, WT control; KO C, KO control; WT T, WT treatment; KO T, KO treatment

Linear Regression Comparison	*F*	P-*value*
WT C – KO C (slope)	0.038	0.845
WT C – KO C (intersect)	1923.66	< **0.0001**
WT C – KO T (slope)	0.009	0.926
WT C – KO T (intersect)	305.296	< **0.0001**
KO C – WT T (slope)	0.894	0.345
KO C – WT T (intersect)	814.799	< **0.0001**

5. Fitness Measures

The social environment has a great impact in the individuals, namely in the fitness such as hierarchy and competition, and success to pass their genes to the next generation (i.e. reproduction success). In order to test how the environment can be responsible for different fitness success in the reproduction of the individuals, different parameters were studied (1) mating success, (2) viability of the embryos, (3) relative gonadal weight.

Regarding the mating success, our results suggest that KO males presented a higher rate of mating success compared to the WT individuals (**Figure 3.6**A) and females that presented a higher rate to mate were KO females raised in WT environment (**Figure 3.6**C).

The results for the male individuals that crossed, revealed that the WT environment besides having a lower mating success, seems to promote a higher viability of the embryos (**Figure 3.6**B). For females, the WT environment besides allowing a higher mating success allows also a higher embryo viability (**Figure 3.6**D).

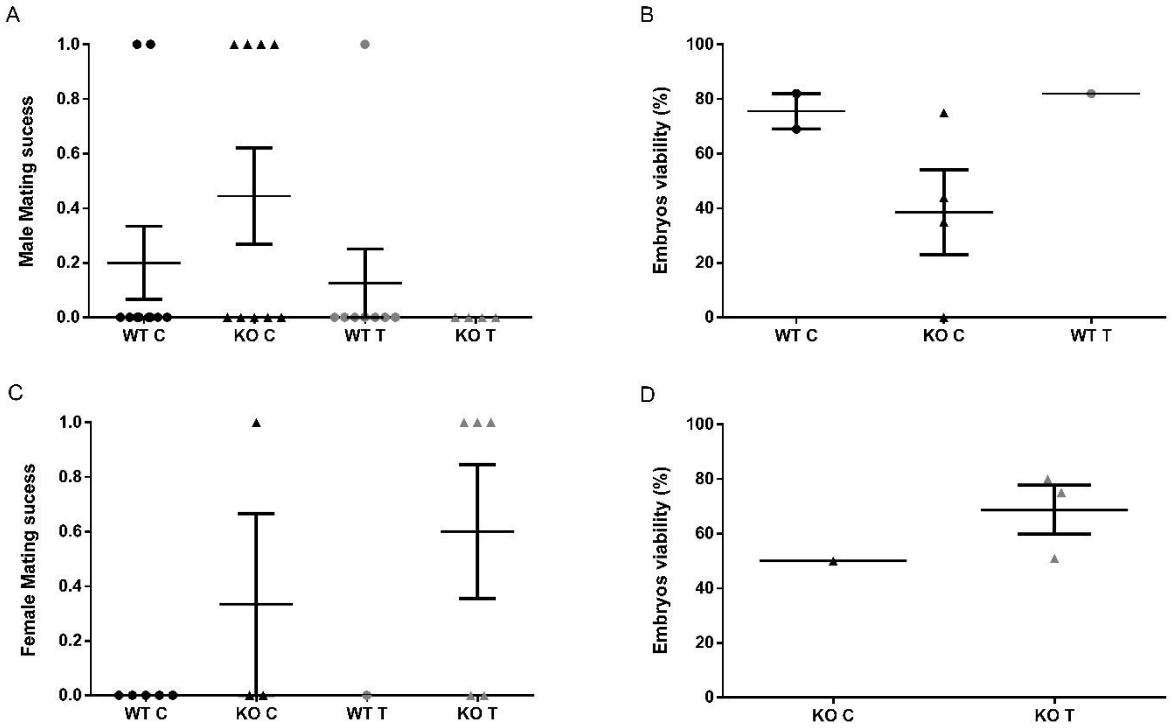

Figure 3.6. Graphical representation of fitness measures, mating success and embryo viability. (**A**, **B**) Male mating success was higher in KO individuals however embryo viability had a higher survival in WT individuals. (**C**, **D**) Female mating success was higher in KO individuals with no crosses with WT and embryo viability had a higher survival in WT environment. WT C, WT control; KO C, KO control; WT T, WT treatment; KO T, KO treatment.

Concerning the relative weight of the male and female gonads, the data revealed a significant difference on the weight of male gonads on the KO environment ($t_{(15)} = 2.714$, $P < 0.05$, **Figure 3.7**), and a significant different on the female gonads on the genotype ($t_{(4)} = 8.033$, $P < 0.01$) and WT environment ($t_{(6)} = 3.278$, $P < 0.05$, **Figure 3.7**). Since it was not possible to control the sex of the focal fish, sample size varied across groups. In the case of the KO environment, statistical analysis was not performed on the female gonads since there was only 1 individual in WT treatment group.

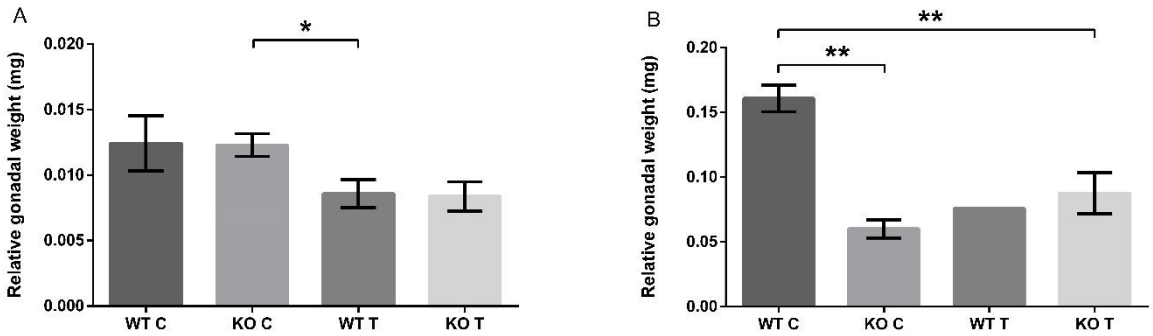

Figure 3.7. Graphical representation of male and female relative gonadal weight. (**A**) Male relative gonadal weight with significant difference on KO environment. (**B**) Female relative gonadal weight was significant on WT genotype and environment. WT C, WT control; KO C, KO control; WT T, WT treatment; KO T, KO treatment. * P < 0.05, ** P < 0.01, *** P < 0.001.

CHAPTER 4 DISCUSSION

Very few studies have focused in studying the impact of social environment, in particular, the effect of nonapeptides on others individuals' phenotypes, and how these interactions modulate behaviours (Bleakley and III 2009; Trubenová and Hager 2014). This study aimed to investigate how social environment, using OxtR mutants, can modulate and develop the behaviour in adult zebrafish.

All fish groups tested were formed at 4 days' larvae to maintain a specific environment with only recognition of the group itself since with 5-6 days' larvae begin to perceive olfactory and visual cues from the environment (Gerlach et al. 2008; Hinz et al. 2013). The phenotyping of adult social behavior was performed between 3-4 months of development, since young adults present a more stable and stronger group cohesion than larvae or juveniles (Buske and Gerlai 2011; Engeszer et al. 2007).

In the shoal preference test, the data revealed an effect of the OXTR mutation, where WT control individuals presented higher shoal preference than KO control group. The data also revealed an effect of mutation where WT control individuals presented higher shoal preference when compared with KO control group (**Figure 3.3**A). This results shown the first insight of indirect genetic effects of oxytocin only for KO genotype, where KO treatment group individuals behave as WT control group (**Figure 3.3**B). The results described above were only significant in the second trial, which can be explain by a novel environment (experimental setup) in first trial, and a habituation to that environment on second trial. From the analysis, the WT environment group when compared with KO environment group have such an impact on shoal preference that it can revert the phenotype in KO individuals. How this happens remains a major question and further work will be needed, however the presence of a second OxtR (OxtR-like) may be the possible route in the transmission of oxytocin making the reversion of phenotype possible. To test this hypothesis, it would be important to generate a double mutant for both receptors hence eliminating any OXT signaling pathway. Double mutants will test the impact of the absence of the receptors. It would also be important to test the effects of the absence of the ligand through the use of transgenic lines. Regarding the environment, the present results suggest that some environments promote higher impact than others, and that they can induce differences in fitness. It is known that the absence of a social environment, social isolation, promotes chronic social behaviour impairments such as a deficit in long-term social recognition, affects locomotor activity and the brain structure (Leser and Wagner 2015; Seid and Junge 2016), social isolation environment that will allow to study the importance of social interactions since they play a key role.

Regarding the data from the social recognition, our test revealed a significant difference between WT and KO individuals independently of the environment, with a strong preference for WT individuals to investigate novel conspecifics. As demonstrated in several other studies (Andersson, Ek, and Olsson 2015; Baracchi et al. 2015; Barba-Escobedo and Gould 2012), the results obtained from social recognition shown that WT individuals only with visual cues can discriminate between familiar and novel conspecifics, which is not observed in KO individuals. This can be explained by the function of Oxt for the normal development of social memory. Once the OxtR is mutated the normal function of the nonapeptide is downregulated or it is not functional. When analyzing the exploration time, the results suggested that fish explore with similar time proportion both tests (novel-novel and novel-familiar; **Figure 3.2**) with a significant reduction of exploratory behaviour along trials, most probably due to habituation. This first data revealed that visual cues alone are enough to form social memory and lead to conspecific recognition.

As an additional result, the performing of three trials revealed insights of long-term memory for WT individuals, again independently from the environment (**Figure S1**). For the first time, this

shown both types of social recognition memory with an inter-trail interval of 48 hours. For WT individuals in the first trial they had a preference for novel conspecifics. In second and third trial, in the Test 1, the preference was already present and maintained in Test 2 (i.e. preference in Test 1 = preference Test 2 in previous trial; **Figure S2**). These results may suggest that long-term memory have a major impact than short-term recognition memory in individuals' interactions and information processing. These results support previous studies in long-term memory for social recognition and novel environment (Barba-Escobedo and Gould 2012; Leser and Wagner 2015). Further work should be done to complement the short vs. long-term memory recognition for instance: (1) performing a social and asocial stimulus preference test using conspecifics and objects respectively to test the capacity of fish to discriminate between these two types of information; (2) to test the capacity of fish to form a long-term memory by expose the focal individuals to the same familiar fish in all trials.

The information that individuals collect in social environment has impact in their fitness and influences their choices. This gather of information is known as social learning and occur in any situation in which one individual observes others and modulate his behaviour in order to optimize his fitness. Regarding the experimental protocol of social learning, if an animal learns the task, i.e. feed in the same location where the demonstrator was eating, it is expected an increase of correct choices and a decrease in the latency to eat. Due to this definition, in a binary task it is expected that the percentage of correct choices to be above 50%. Because this was not observed the fish were not learning. All animals from the simplest to the most complex, live according to the cost-benefit criteria (trade-off) in which the choices influence their development, their fitness and the probability of getting food or not. Since in the experimental setup the stimulus fish were in the same side of the tank and relatively closer to each other, the cost-benefit to go to one side or to the other were much less the same, however if the stimulus were in opposite sides the costs of the choice would be bigger. Use stimulus fish in opposite sides should confer a higher trade-off for the focal fish leading to better learning performance learning.

Zebrafish in the past years emerged as a new model species for the study of the neurobiology of social behaviour, given its social nature and the innumerous molecular tools available to visualize and manipulate neural circuits in this species. Gerlai and colleagues shown in three main studies one of the typical characteristics of zebrafish that is shoaling, which present temporal oscillations, is age-dependent with an increase of shoaling with age, and is influence by changes in the environment (Buske and Gerlai 2011; Miller and Gerlai 2007, 2008). Our results support Gerlai studies which revealed that oxytocin by itself can modulate group cohesion (**Figure 3.5**): Oxt mutants form more cohesive groups than WT, and this can result from the absence of OxtR, since it is known that oxytocin modulate sociality and anxiety levels in zebrafish (Braida et al. 2012). The results from this test also revealed that in the KO treatment group, the shoal was more disperse. However, since group cohesion is a group, and not an individual, measure the results demonstrate the global effect of the social environment in the group and not at the individual level. Regarding the WT treatment group, the shoal was more disperse, which can indicate a reverse of environment social behaviour and thus the data suggest than one individual is enough to modulate the entire group, indicating social selection which assumes that a given phenotype in one individual affects the fitness of other individuals directly (Trubenová and Hager 2014). To conclude, like shown in others studies not only in zebrafish but also in mice, reptiles, and in other animals (Chapman and Krause 2008; Engeszer et al. 2007; Leal and Powell 2012; Miller and Gerlai 2007; Seid and Junge 2016), the environment is a crucial factor in the development and modulation of social behaviour and different environments promote different individual fitness.

The environment can have profound effects on the individual fitness that range from modification of social behaviour to physiological alterations, such as investment in reproduction. To test the impact of social environment in such characteristics mating success, embryos viability and relative gonadal weight were measured. The data shown that WT males presented a lower mating

success but their embryos viability was higher. In females, only KO individuals crossed in both control and treatment groups, although it was the WT environment that promoted the highest viability of the embryos. These results suggest that the investment in reproduction is different between different genotypes, where regarding male KO, the higher mating success was due by the necessity of higher investment to pass the genes to next generation even if it causes a lower embryo quality. For females, the results shown the importance of environment and how this can influence the investment of reproduction in which females KO in a WT environment presented a higher mating success and embryo viability. Regarding the WT females this shown that they are more environment-dependent due to the fact that the same genotype can lead to different reproduction investments. While in males, independently from the environment the same genotype, WT individuals, had similar mating success and embryo viability. Although, further work is needed to increase the number of animals per group.

Finally, male and female gonad weights revealed that it was the females in the WT control group that invested more in reproduction with only a difference in male gonads, where environment leads to a reduction of gonadal weight for WT treatment group individuals.

CHAPTER 5 CONCLUSION

In conclusion, the present work had shown for the first time the importance of social environment (i.e. the genotype of the group) in the development of behaviour, mainly how a single mutation can change the behaviour and how social environment can revert the mutants' phenotype.

We also demonstrated, through the use of oxytocin mutants, an impairment in the cognitive abilities which had also been shown in mice (Winslow and Insel 2002). So, this study contributes more to the literature in a way that shows the importance of the oxytocin-like peptides in the behaviour and in cognition.

This study gave us many conclusions regarding behaviour and also how behaviour influences the dynamic of a group and the reproduction capacity. These results demonstrated that the genetic social environment plays an important role in individuals' fitness and for the first time that oxytocin is an important neuropeptide in all cognitive abilities which can influence the individuals in a group.

All these procedures, opens a lot of windows to explore. As the results shown the effect of the environment in the development of behaviour in zebrafish future work should be done to answer different questions. If environment influences the behaviour of oxytocin knockouts individuals, what happens in the brain? Is there a modification of transcription, a second pathways of oxytocin signaling? To test this hypothesis immunostaining for oxytocin should be done and the number of cells counted, and also some brains should be collected and gene expression levels analyzed.

Because the presence of a second OxtR, the use of double mutants for the receptor and the use of a transgenic line for the ligand should be important to test the effect of both in the behaviour and how environment will influence the individuals.

As described in thorough the present work, environment has an important role in the social interactions but how social isolation influence the behaviour? Doing an isolation group, where individuals spend early stages of development in social isolation will gave us an extreme environment where a lack of social cognition skills is expected. If environment would lead to such extreme behaviour how the progeny will be influenced by the parental exposure to the environment? Different studies of transgenerational epigenetic effects suggest a transmission of information from parents to their progeny. Crossing fish that grow in isolation with fish that grown in regular groups will show as the transgenerational effects in the following generations.

As a final remark, more studies should be done to see the impact of the environment in the individuals and also how genetics promotes such huge variability in behaviour.

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SUPPLEMENTARY MATERIAL

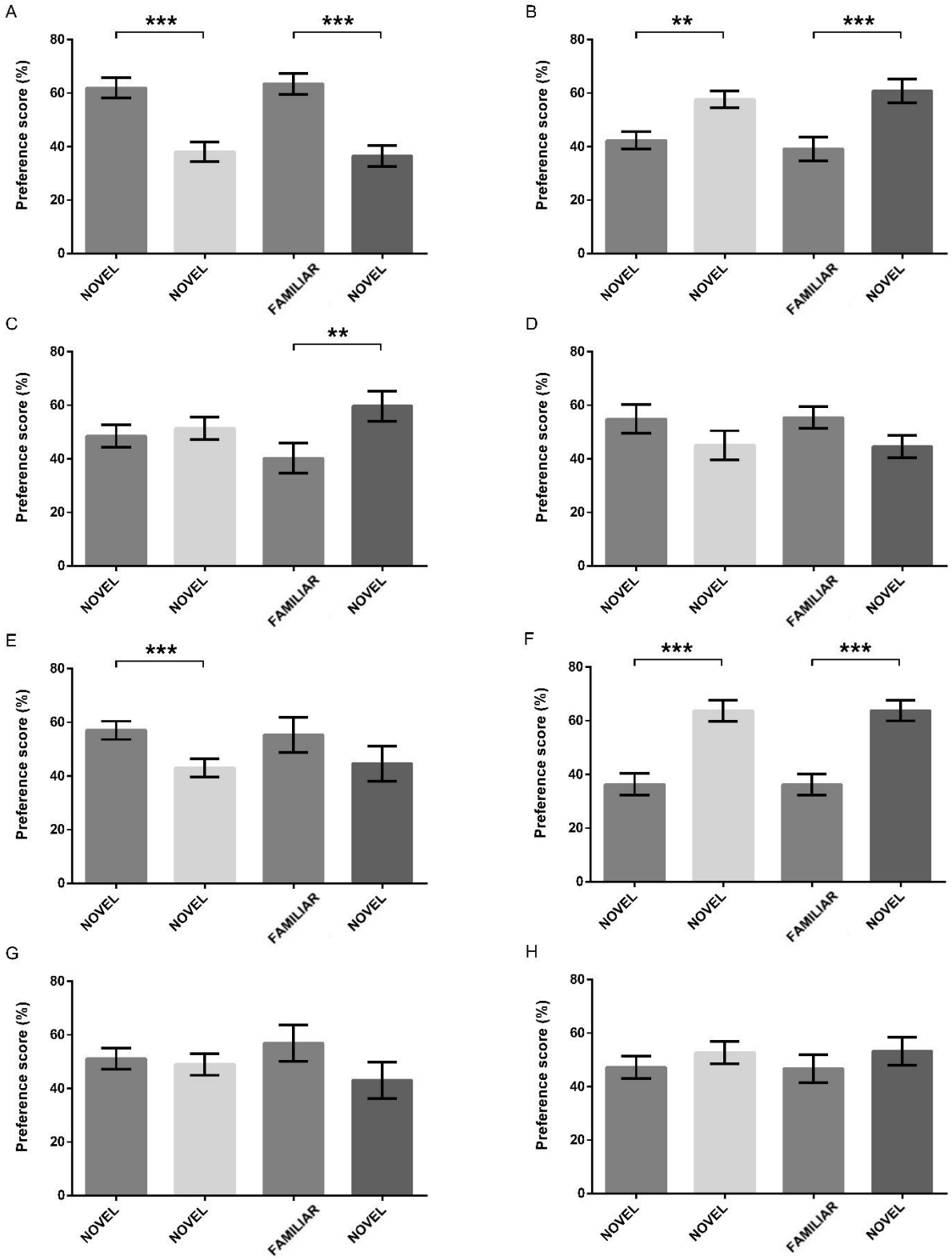

Figure S1. Graphical representation of social recognition behavioural test on trial 2 and 3. Preference scores of the time spent near novel-novel (Test 1) or novel-familiar (Test 2) conspecifics. Error bars represent as mean ± SEM. (**A**, **B**) OxtR WT control group in Test 1 presented already a preference spending signicantly more time near the side where in the previous trial he presented a higher preference score indicating a long-term memory. (**C**, **D**) OxtR KO control group do not present preference in Trial 2 and 3 but discriminate between novel and familiar on trial 2. (**E**, **F**) OxtR WT control group in Test 1 presented already a preference spending signicantly more time near the side where in the previous trial he presented a higher preference score indicating a long-term memory. (**D**) OxtR KO treatment group do not discriminate between novel and familiar conspecifics (n = 9 per treatment). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Table S1. Effect of social environment on Test 1 of social recognition task. Main effects, interactions and planned comparisons of LS means were calculated using Repeated measures ANOVA. WT C, WT control; KO C, KO control; WT T, WT treatment; KO T, KO treatment.

Effect (Novel – Novel)		*F*	*P-value*
Genotype		0	1
Test		2.338	0.13
Genotype * Test		2.084	0.108
Trials		0	1
Trials * Genotype		0	1
Trials * Test		8.593	< 0.001
Trials * Genotype * Test		4.133	< 0.01
Planned Comparisons (Novel – Novel)			
WT C	TRIAL 1	1.316	0.255
	TRIAL 2	22.223	< 0.001
	TRIAL 3	7.207	< 0.01
KO C	TRIAL 1	0.018	0.895
	TRIAL 2	0.312	0.578
	TRIAL 3	2.982	0.088
WT T	TRIAL 1	1.949	0.166
	TRIAL 2	4.587	< 0.05
	TRIAL 3	13.839	< 0.001
KO T	TRIAL 1	1.319	0.254
	TRIAL 2	0.105	0.746
	TRIAL 3	0.57	0.452

Table S2. Effect of social environment on Test 2 of social recognition task. Main effects, interactions and planned comparisons of LS means were calculated using Repeated measures ANOVA. WT C, WT control; KO C, KO control; WT T, WT treatment; KO T, KO treatment.

Effect (Novel – Familiar)		*F*	*P-value*
Genotype		0	1
Test		8.002	< 0.01
Genotype * Test		2.572	0.059
TRIALS		0	1
TRIALS * Genotype		0	1
TRIALS * Test		8.584	< 0.001
TRIALS * Genotype * Test		7.035	< 0.001

Planned Comparisons (Novel – Familiar)

WT C	TRIAL 1	10.97	< 0.01
	TRIAL 2	14.744	< 0.001
	TRIAL 3	14.454	< 0.001
KO C	TRIAL 1	2.134	0.148
	TRIAL 2	7.641	< 0.01
	TRIAL 3	3.653	0.059
WT T	TRIAL 1	6.947	< 0.05
	TRIAL 2	1.398	0.24
	TRIAL 3	14.061	< 0.001
KO T	TRIAL 1	0.063	0.803
	TRIAL 2	2.347	0.129
	TRIAL 3	0.774	0.382

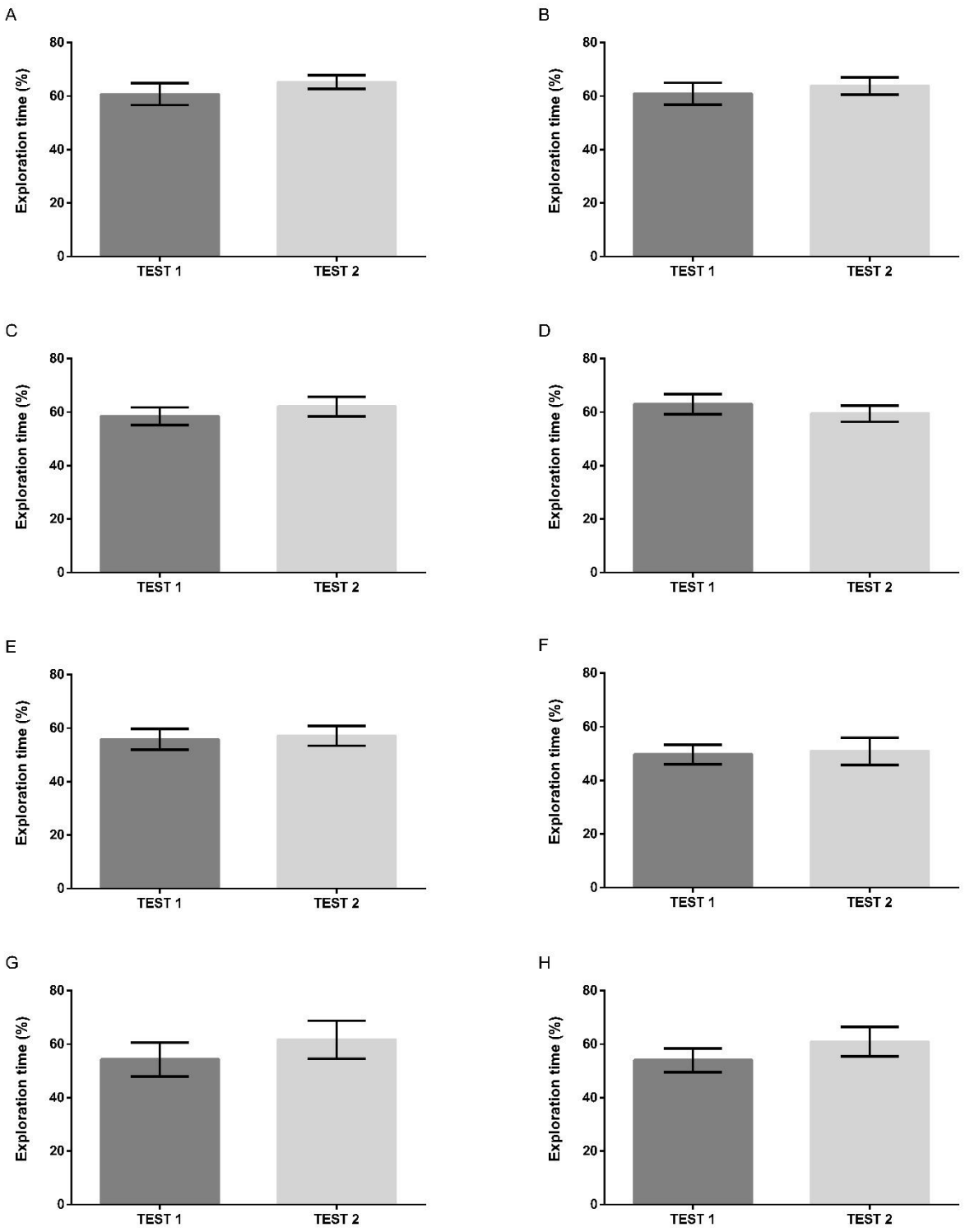

Figure S2. Graphical representation of exploration time on trial 2 and 3. Exploration time near each ROI during Test 1 (novel-novel) and Test 2 (novel-familiar). Error bars represent as mean ± SEM. In Test 1 and 2 no differences between exploration time was observed in the last two trials. (**A**, **B**) OxtR WT control (n = 15 per treatment). (**C**, **D**) OxtR KO control group (n = 15 per treatment). (**E**, **F**) OxtR WT treatment group (n = 9 per treatment). (**G**, **H**) OxtR KO treatment group (n = 9 per treatment). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Table S3. Effect of social environment on exploration task in the three trials performed. Main effects and interactions were calculated using Repeated measures ANOVA.

Effect (Test 1 – Test 2)		*F*	*P-value*
Environment		1.037	0.38
Test		0.501	0.481
Environment * Test		0.206	0.892
TRIALS		4.315	< 0.05
TRIALS * Environment		3.11	< 0.01
TRIALS * Test		1.445	0.238
TRIALS * Environment * Test		0.413	0.87
Planned Comparisons (Test 1 – Test 2)			
WT C	TRIAL 1	0.048	0.827
	TRIAL 2	0.738	0.393
	TRIAL 3	0.318	0.575
KO C	TRIAL 1	0	1
	TRIAL 2	0.46	0.5
	TRIAL 3	0.476	0.492
WT T	TRIAL 1	0.348	0.557
	TRIAL 2	0.036	0.85
	TRIAL 3	0.033	0.857
KO T	TRIAL 1	0.242	0.624
	TRIAL 2	1.146	0.287
	TRIAL 3	1.101	0.297